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RESEARCH ARTICLE

Oxygen consumption remains stable while ammonia excretion is reduced upon short time exposure to high salinity in *Macrobrachium acanthurus* (Caridae: Palaemonidae), a recent freshwater colonizer

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ABSTRACT. Palaemonid shrimps occur in the tropical and temperate regions of South America and the Indo-Pacific, in brackish/freshwater habitats, and marine coastal areas. They form a clade that recently (i.e., ~30 mya) invaded freshwater, and one included genus, *Macrobrachium* Bate, 1868, is especially successful in limnic habitats. Adult *Macrobrachium acanthurus* (Wiegmann, 1836) dwell in coastal freshwaters, have diadromous habit, and need brackish water to develop. Thus, they are widely recognized as euryhaline. Here we test how this species responds to a short-term exposure to increased salinity. We hypothesized that abrupt exposure to high salinity would result in reduced gill ventilation/perfusion and decreased oxygen consumption. Shrimps were subjected to control (0 psu) and experimental salinities (10, 20, 30 psu), for four and eight hours (n = 8 in each group). The water in the experimental containers was saturated with oxygen before the beginning of the experiment; aeration was interrupted before placing the shrimp in the experimental container. Dissolved oxygen (DO), ammonia concentration, and pH were measured from the aquaria water, at the start and end of each experiment. After exposure, the shrimp's hemolymph was sampled for lactate and osmolality assays. Muscle tissue was sampled for hydration content (Muscle Water Content, MWC). Oxygen consumption was not reduced and hemolymph lactate did not increase with increased salinity. The pH of the water decreased with time, under all conditions. Ammonia excretion decreased with increased salinity. Hemolymph osmolality and MWC remained stable at 10 and 20 psu, but osmolality increased (~50%) and MWC decreased (~4%) at 30 psu. The expected reduction in oxygen consumption was not observed. This shrimp is able to tolerate significant changes in water salt concentrations for a few hours by keeping its metabolism in aerobic mode, and putatively shutting down branchial salt uptake to avoid massive salt load, thus remaining strongly hyposmotic. Aerobic metabolism may be involved in the maintainance of cell volume, concomitant with reduced protein/aminoacid catabolism upon increase in salinity. More studies should be conducted to broaden our knowledge on palaemonid hyporegulation.

KEY WORDS. Ammonia, lactate, osmoregulation, palaemonidae.

INTRODUCTION

Palaemonid shrimps have a wide global distribution. They occur in a great variety of aquatic environments, from seawater up to full freshwater (Augusto et al. 2009, Anger 2013). The family Palaemonidae has a marine origin and passed through various independent events of freshwater invasion (Murphy and Austin 2005, Ashelby et al. 2012, McNamara and Faria 2012, Anger 2013). These events have occurred quite recently, ~30 mya (Tertiary), and are still happening (Murphy and Austin 2005, Augusto et al. 2007a, b, 2009, Pileggi and Mantelatto 2010, Collins et al. 2011, McNamara and Faria 2012). The family comprises about 116 genera. Among these, *Macrobrachium* Bate, 1868 was the most successful colonizer of freshwater and estuarine waters, showing a wide geographical distribution (Anger 2013). One species in particular, commonly referred to as "giant

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freshwater prawn," *Macrobrachium rosenbergii* (De Man, 1879) has large economic importance globally (Bond-Buckup and Buckup 1989, Valenti et al. 1989).

The fact that freshwater Palaemonid shrimps have recently transitioned from saline waters to more dilute waters renders them quite tolerant to increased salinity (Freire et al. 2008a, 2013). Freshwater Palaemonid shrimp adults - especially the more coastal and diadromous species – those whose larvae depend on brackish waters for their proper development (Charmantier 1998, Anger 2003, McNamara and Faria 2012), are particularly euryhaline (McNamara 1987).

Estuarine, but especially freshwater crustaceans, are good hyper-osmoregulators, that is, they keep steep osmotic and ionic gradients with respect to the surrounding water, aided by the low permeability of their cuticle (Péqueux 1995, Freire et al. 2003, 2008a, b). Palaemonid shrimps also follow this pattern (Freire et al. 2003, Murphy and Austin 2005, Augusto et al. 2009, Boudour-Boucheker et al. 2013). It is during ecdysis that cell volume may be challenged in these shrimps, as their internal medium fluctuates (compared to the intermoult period), diluting strongly in fresh waters. However, crustaceans in general, palaemonid shrimps in particular, can face this challenge by regulating cell/tissue hydration quite efficiently (see Freire et al. 2013). These hyper-regulation mechanisms, one of the most prominent features of freshwater crustaceans (second to the cuticle) have been frequently studied in Palaemonidae (e.g., Freire et al. 2008b, McNamara and Faria 2012). Hyporegulation mechanisms, by contrast, have been much less investigated and remain elusive (Freire et al. 2008b, McNamara and Faria 2012).

Salinity challenges can also result in changes in metabolic responses. For instance, a decrease in the respiratory rate of the marine shrimps *Marsupenaeus* (*Penaeus*) *japonicus* (Bate, 1888) (Setiarto et al. 2004), and *Litopenaeus (Penaeus) setiferus* (Linnaeus, 1767) (Rosas et al. 1999), when exposed to a decrease in salinity, was observed. Some palaemonid shrimps exposed to salinity increases, for instance *Macrobrachium heterochirus* (Wiegmann, 1836) and *Macrobrachium potiuna* (Müller, 1880), experience a decrease in metabolic rates, while the diadromous *Macrobrachium acanthurus* (Wiegmann, 1836) and *Macrobrachium olfersii* (Wiegmann, 1836) experience a peak in their metabolism-salinity curves ("dome-shaped curve") close to their isosmotic point, ~21 psu (Moreira et al. 1983). In another diadromous shrimp, *Macrobrachium amazonicum* (Heller, 1862), oxygen consumption was lower in freshwater than in 18 psu in zoea II, and was higher in freshwater than in 12 and 18 psu in zoea V (Mazzarelli et al. 2015). The freshwater shrimp *Macrobrachium tuxtlaense* Villalobos and Alvarez, 1999, when exposed to increased salinities up to 30 psu, has shown an increase in oxygen consumption in 5 and 10 psu, and a decrease in this parameter in the other salinities, with respect to the control salinity, fresh water (Ordiano et al. 2005). With this variability in the metabolic response of shrimps in the background, the aim of this study was to test whether a short term exposure to increased salinity would result in a "shut down"of oxygen uptake, a putative "escape response", potentially activating anaerobic metabolism and lactate production in this diadromous palaemonid.

MATERIAL AND METHODS

Specimens of *M. acanthurus* were bought from local fishermen from Rio dos Barrancos (25°36'32.0"S, 48°24'02.5"W), municipality of Pontal do Paraná, Paraná, Brazil, who sell them as live bait. Shrimps were transported to the laboratory for approximately two hours, in plastic gallons with constant aeration. The animals were acclimated for about five days in 35 liters aquaria with fresh water (double filtered tap water, charcoal and cellulose filters), in temperature of 20±1°C, constant aeration, and natural photoperiod (~12 h light: 12 h dark). Some ions were assayed in our tap water (mean±standard deviation, in mM, n = 6 for all): chloride 0.23 ± 0.29; magnesium: 0.16 ± 0.06; sodium 4.67 ± 1.94; potassium 0.57 ± 0.20, and osmolality of 26.2 ± 4.3 mOsm/kg H_2O. Shrimps were fed fragments of fish fillet on alternate days.

Shrimps (5.2 ± 0.7 cm, n = 64) were individually subjected to salinities 0 (control), 10, 20, or 30 psu, for 4 or 8 hours (n = 8 for each coupled condition of salinity x time), in 250 ml containers, water temperature of 21.1 ± 0.05 °C. Saline waters were obtained through proportional mixture of filtered tap water with natural sea water. The experiments were conducted without aeration, in order to allow the determination of oxygen consumption by the shrimp, but the initial water was saturated with oxygen, through overnight aeration, before the start of the experiments (initial oxygen concentration of 7.49 ± 1.19 mg/l for 0 psu, 7.36 ± 0.60 mg/l for 10 psu, 6.73 ± 0.15 mg/l for 20 psu, and 6.55 ± 0.15 mg/l for 30 psu, n = 16 for each salinity). The following water parameters were analyzed at the initial (before placing the shrimp in the container) and final (after removing the shrimp from the container) times of exposure: dissolved oxygen, pH, and ammonia. Differences between the initial and final concentrations of dissolved oxygen and ammonia represented, respectively, oxygen consumption and ammonia excretion by the shrimp ($N\text{-}NH_3$). There was essentially no ammonia in the water at the beginning of the experiments (0.008 ± 0.003 mg/l of $N\text{-}NH_3$, n = 70 samples). Experiments were also conducted in containers with water but without animals, as blanks for water parameters (n = 6 for each experimental condition, yielding a total of 72 containers).

After the stipulated times of exposure, the animals were cryoanesthetized (covered with ground ice) for about 1 minute, until fully immobile. Then, hemolymph samples were collected through cardiac puncture, with a micropipette inserted under the exosqueleton, for determinations of lactate and osmolality. Finally, the exosqueleton was removed, and a fragment of abdominal muscle was collected for determination of water content.

Other individuals were subjected to the same protocol of salinity increase (n = 3 for each condition of salinity x time), to evaluate whether shrimps were ventilating their gills equally

in high salinity media, as they do in their habitat, fresh water. The hypothesis was that their gills would get stained from the dye added to the water, after some minutes of exposure, from gill ventilation. At the end of the experimental exposures to high salinity, five drops of 1% methylene blue were added to each of the 250 ml containers. Shrimps were maintained in these conditions for 5 minutes, after which they were removed from the containers and had both sides of their cephalotorax photographed, with focus on their gills. The intensity of the blue staining of their gills was qualitatively evaluated. The same procedure with the dye was conducted for control shrimps in fresh water (see Suppl. material 1).

The levels of dissolved oxygen were detected in the water through an oxymeter (YSI model 55, USA). Water pH was determined using a bench pHmeter (inoLAB pH Level 1WTW, Germany). The concentration of ammonia was assayed through colorimetric commercial kits (Alfakit, Brazil), and absorbance was read at 630 nm (Spectrophotometer Ultrospec 2100 PRO Amersham Pharmacia biotech, Sweden).

Hemolymph lactate was assayed through colorimetric commercial kits (Labtest, Brazil), with absorbance read at 550 nm. Hemolymph osmolality was determined using a vapour pressure osmometer in undiluted samples (Vapro 5520, Wescor, USA). For the determination of muscle water content, tissue fragments were weighed (wet weight, analytical balance Bioprecisa FA2104N, Brazil, precision of 0.1 mg), dried in an oven at 60 °C for 24 hours, then weighed again (dry weight). The difference between wet and dry weights, as a percentage, represents the muscle water content, or its hydration.

Two-way ANOVAs (factors were salinity and time) with *post hoc* tests of Holm-Sidak were conducted for each of the following parameters: oxygen consumption, lactate, osmolality, and muscle water content. Initial and final pH values did not pass the normality and equal variance tests. These data were transformed to meet the requirements of the parametric two way ANOVA. Ammonia values could not be normalized, and for this reason they were treated differently. Two non-parametric (Kruskal-Wallis) "one-way-ANOVAs" were conducted, one for 4 hours, one for 8 hours. The respective values of each salinity, 4 vs 8 hours, were compared using t-tests. The initial versus final values of dissolved O_2, and pH in the water of containers were compared through paired t-tests for each experimental condition. Pearson correlations were performed for factors salinity, oxygen consumption, lactate, osmolality, muscle water content, excreted ammonia, final pH, and total length. The adopted significance level was 0.05.

RESULTS

Experimental blanks for water parameters

The initial and final water parameters (O_2, pH, and NH_3) in the blanks, vials without any shrimp – for all salinities and times of exposure – are shown in Table 1.

Table 1. Initial and final concentrations of oxygen and ammonia, and values of pH in the water of "blank" containers, without any shrimp, in salinities 0 (control), 10, 20, and 30 psu, for 4 and 8 hours of exposure (n = 6 for each group).

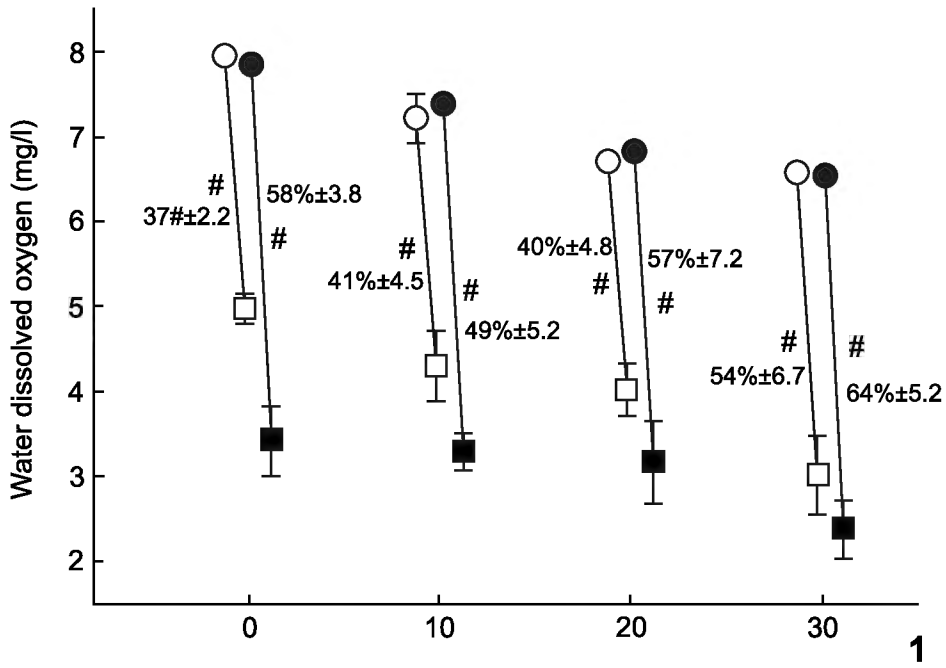
	Initial	After 4 hours	After 8 hours
O_2 (mg/l)			
0 psu	6.88 ± 0.41	6.89 ± 0.24	6.74 ± 0.16
10 psu	6.98 ± 0.21	6.84 ± 0.12	6.69 ± 0.05
20 psu	6.63 ± 0.19	6.48 ± 0.10	6.27 ± 0.04
30 psu	6.29 ± 0.09	6.08 ± 0.03	5.85 ± 0.02
pH			
0 psu	6.90 ± 0.16	7.05 ± 0.15	6.88 ± 0.09
10 psu	7.61 ± 0.05	7.47 ± 0.04	7.42 ± 0.02
20 psu	8.03 ± 0.06	7.81 ± 0.05	7.71 ± 0.05
30 psu	8.24 ± 0.03	8.05 ± 0.05	7.93 ± 0.05
NH_3 (mg/l)			
0 psu	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.01
10 psu	0.01 ± 0.01	0.01 ± 0.01	0.01± 0.01
20 psu	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
30 psu	0.00 ± 0.01	0.01 ± 0.01	0.01 ± 0.01

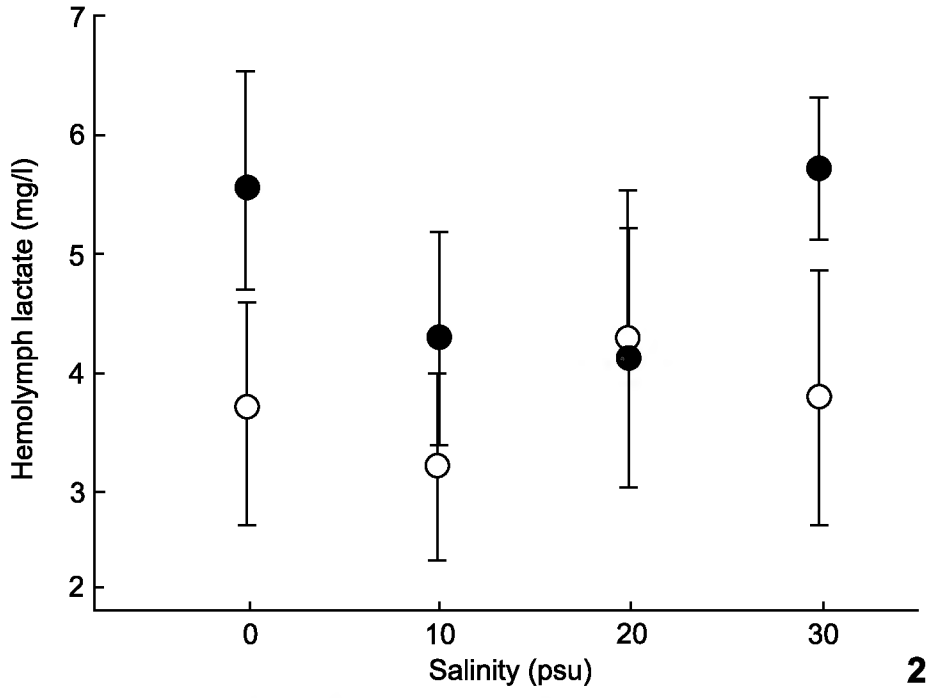
Water oxygen consumption and lactate concentration in the hemolymph

The two-way ANOVA revealed that time ($F = 14.1$, $p < 0.001$) and salinity ($F = 2.9$, $p = 0.043$), but not their interaction ($F = 0.66$, $p = 0.58$) affected water oxygen consumption in *M. acanthurus*. The initial oxygen concentration in the water was always higher than the final concentration, for all salinities and times of exposure, indicating oxygen consumption by the shrimp (Fig. 1). However, consumption, as quantified by the difference between initial and final oxygen levels in the water of the container, was stable and did not vary among the experimental treatments and controls. The two-way ANOVA revealed that time ($F = 4.3$, $p = 0.042$), but not salinity ($F = 0.068$, $p = 0.977$), or their interaction ($F = 0.15$, $p = 0.93$) affected hemolymph lactate in *M. acanthurus*. Production of lactate by the shrimp, measured from its hemolymph, was essentially invariable in the several experimental conditions (Fig. 2).

pH and excreted ammonia

The two-way ANOVA on initial pH values revealed no effect of time ($F = 0.79$, $p = 0.38$), but an effect of salinity ($F = 132$, $p < 0.001$), and no interaction between time and salinity ($F = 0.82$, $p = 0.49$). The final pH of the water , according to the two-way ANOVA, was affected by time ($F = 19.5$, $p < 0.001$), and salinity ($F = 56.3$, $p < 0.001$), but not by their interaction ($F = 1.100$, $p = 0.36$). The pH of the water was always higher at the start (initial) of the experiment than after 4 and 8 hours (final, Fig. 3). The Kruskal-Wallis tests revealed that salinity had an effect on water ammonia concentrations after 4 hours ($p < 0.001$), and after 8 hours ($p < 0.001$). The amount of excreted





Figures 1–2. Initial (circles) and final (squares) levels of dissolved oxygen in the water (1) and lactate concentration in the hemolymph (2) of *M. acanthurus* exposed to salinities 0 (control), 10, 20, and 30 psu for 4 (white symbols) and 8 hours (black symbols). Values near to the lines represent oxygen consumption (mean ± std dev) as a percentage of the initial oxygen concentration (considered 100%, as a reference value). (#) Initial and final levels of dissolved oxygen are different. There were no significant differences between salinities or times (4 and 8 hours) for oxygen consumption and lactate concentration.

ammonia ($N\text{-}NH_3$) decreased with increasing salinity, with the highest value in 0 and 10 psu, and the lowest values in 20 and 30 psu. The effect of time on the amount of excreted ammonia was noted in the controls in 0 only, with higher ammonia levels measured in the water after 8 hours than after 4 hours (Fig. 3).

Osmolality of the hemolymph and muscle water content

The two-way ANOVA revealed that time ($F = 12.9$, $p < 0.001$) and salinity ($F = 79.8$, $p < 0.001$), and their interaction ($F = 7.3$, $p < 0.001$) had an effect on the osmolality of the hemo-

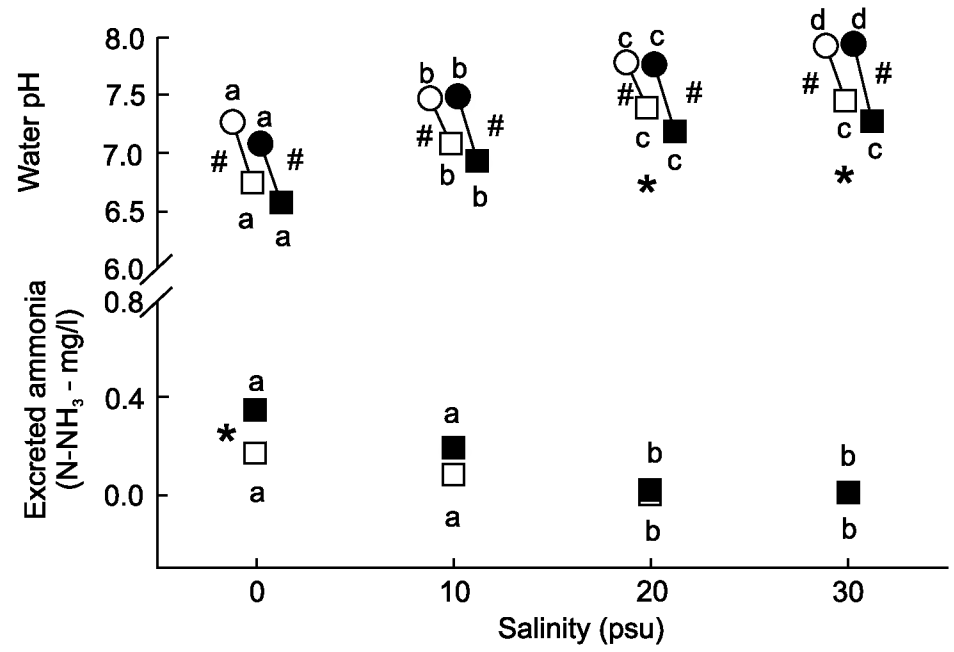


Figure 3. Initial (circles) and final (squares) pH and excreted ammonia ($N\text{-}NH_3$) in water of *M. acanthurus* exposed to salinities 0 (control), 10, 20, and 30 psu for 4 (white symbols) and 8 hours (black symbols). Different letters mean differences between salinities within each time of exposure. * = value for 4 hours is different from value for 8 hours within a same salinity, # = initial and final values of water pH are different.

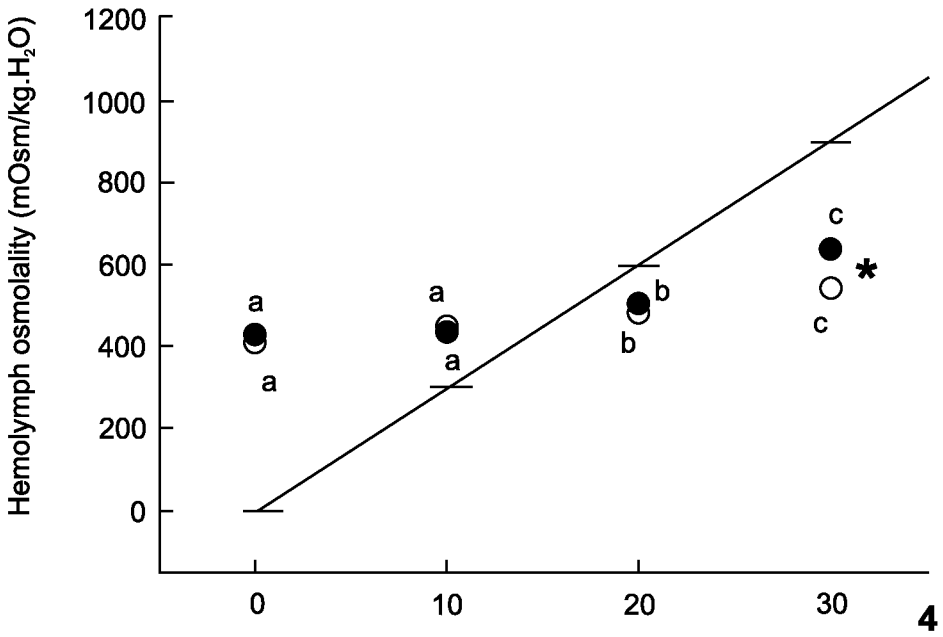
lymph of *M. acanthurus*. It increased by 20 psu, and further by 30 psu with respect to the control (0 psu), after 4 and 8 hours. An effect of time was noted at 30 psu: osmolality was higher after 8 hours than after 4 hours (Fig. 4). Coherently, muscle water content showed the opposite trend, that is, it decreased as salinity increased. The two way ANOVA revealed that salinity ($F = 25.3$, $p < 0.001$) and the interaction between salinity and time ($F = 2.95$, $p = 0.041$) affected the hydration of the muscle, but time alone did not ($F = 2.3$, $p = 0.14$). In the highest salinity, 30 psu, muscle water content after 8 hours was lower than after 4 hours, matching the raised osmolality of the hemolymph (Fig. 5).

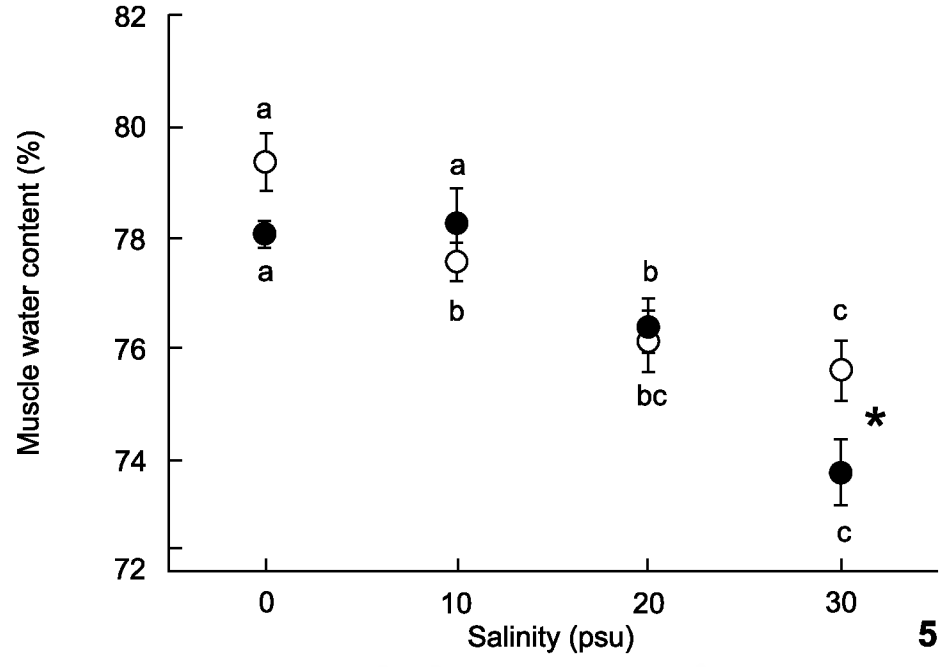
Pearson correlation

Salinity had a positive correlation with oxygen consumption (Weak Correlation coefficient 0.254, P value 0.0430), with water pH (Strong Correlation coefficient 0.808, P value $7.54x10^{-16}$), and osmolality (Strong Correlation coefficient 0.813, P value $3.53x10^{-16}$). Conversely, salinity had a negative correlation with excreted ammonia (Strong Correlation coefficient -0.775, P value $6.02x10^{-14}$) , and muscle water content (Strong Correlation coefficient -0.72, P value $1.82x10^{-11}$).

DISCUSSION

Water dissolved oxygen (DO) was consumed by *M. acanthurus* during the experiments, and was consistently detected by our assay method, which is evidenced by the reduction in water DO. No decrease in water DO was detected under the same experimental conditions (volume, temperature, previous





Figures 4–5. Hemolymph osmolality (4) and muscle water content (5) of *M. acanthurus* exposed to salinities 0 (control), 10, 20, and 30 psu for 4 (white circles) and 8 hours (black circles). Dashed line represents water expected values, from the relationship 1 psu = 30 mOsm/kg H_2O, short horizontal lines indicate value of calculated water osmolality for the tested salinities. Different letters mean differences between salinities within each time of exposure. * = value for 4 hours is different from value for 8 hours within a same salinity.

DO saturation protocol, DO electrode), but without a shrimp in the vial ("blanks"), supporting the conclusion that oxygen was indeed consumed by the shrimp. Unexpectedly, salinity did not affect oxygen consumption by *M. acanthurus* after 4 or 8 hours of exposure; the correlation between these two variables was significant, but weak.

The relationship between salinity and oxygen consumption in shrimps is rather complex and variable. In the marine palaemonid *Palaemon serratus* (Pennant, 1777) no change in oxygen consumption between salinities 34 and 15 psu (Salvato et al. 2001) was observed. In contrast, in the marine shrimp *L.* (*P.*) *setiferus* the effect of salinity on the rate of oxygen consumption was influenced by the developmental stage of the individual: post-larvae PL10-PL15 showed the highest oxygen consumption at 10 psu, while post-larvae PL15-PL21 showed the highest oxygen consumption at 40 psu (salinity ranged from 5 to 40 psu – Rosas et al. 1999).

Importantly, in the study cited immediately above (Rosas et al. 1999), oxygen consumption in penaeid shrimps was measured in these cited salinity levels after 120-264 hours of exposure to them. In addition, penaeid marine shrimps are hyper-hypo-regulators (Péqueux 1995, Freire et al. 2008b), as opposed to palaemonid shrimps, which are essentially hyper-regulators, as already mentioned. In the results of an experiment with *M. amazonicum* at different ontogenetic stages (zoea I, II, V, and IX) exposed to different salinities (0.5, 6, 12 or 18 psu), there was a greater consumption of oxygen at 0.5 psu in the zoea stage V, probably due to the great amounts of energy required for the active transport of salts through the epithelia (Mazzarelli et al. 2015). A similar pattern was observed in *M. tuxtlaense*, an strictly freshwater prawn exposed to a salinity gradient (0, 5, 10, 15, 20, 25, and 30 psu): higher rates of oxygen consumption in the shrimp *M. tuxtlaense* were observed at low salinities (0, 5 and 10 psu), to account for hyper-regulation of the osmolality of the hemolymph (Ordiano 2005). In the results of Moreira et al. (1983) working with *M. acanthurus*, the oxygen consumption curve after 24 hours of exposure was dome-shaped, peaking at 21 psu. These results show that the metabolic response to variations in salinity is indeed variable in these decapod crustaceans, is dependent on the osmoregulatory history and strategy of the species, and is also time-dependent.

Consistent with the results on oxygen consumption rates, the concentration of hemolymph lactate in *M. acanthurus* remained constant, they did not change when salinity increased. The hypothesis here was that increased salinity would lead to a reduction in gill perfusion and, consequently, reduced oxygen consumption. Reduced oxygen consumption would result in anaerobic metabolism and lead to increased levels of hemolymph lactate (Booth et al. 1982). In many decapod crustaceans, lactate is the main product of metabolism when there is a hypoxic condition (Bridges and Brand 1980, Taylor and Spicer 1989, Maciel et al. 2008). These shrimps continued to take up oxygen from the water, and did not enter into functional hypoxia. Had they entered into intense anaerobic metabolism, increased hemolymph lactate would have been detected. However, metabolic carbon dioxide was produced and released through the gills, causing water acidification (Henry and Wheatly 1992), which was detected in our results.

Ammonia release decreased with increased salinity (strong and significant negative Pearson correlation). One possible factor that could at least partially account for this inverse relationship is the fact that NH_3 can be excreted as NH_4^+, especially in acidic water, and in animals with acidosis, replacing K^+ in the Na^+/K^+-ATPase (e.g., Claiborne et al. 1982, Wall 1995, Furriel et al. 2004). If Na^+/K^+-ATPase activity is reduced with increased salinity in this shrimp (see Maraschi et al., 2015), then NH_4^+ transport from the hemolymph also putatively decreases, leading to less

ammonia in the water, as observed here. Alternatively, ammonia may also be transported and eliminated through Rh-proteins (see Weirauch et al. 2009).

This freshwater shrimp strongly hyper-regulates in freshwater (gradient of +400 mOsm/kg H_2O), its natural habitat in the adult phase, and continues to show hyper-osmotic hemolymph after 4-8 hours in 10 psu (+100 mOsm/kg H_2O). However, after 4-8 hours in 20 or 30 psu, although there is some increase in hemolymph osmolality (strong positive correlation between salinity and hemolymph osmolality), it becomes hyposmotic to the water at -150 and -300 mOsm/kg H_2O, respectively.

When there is a significant salt load, for instance 10, 20, 30 psu, what happens to the salt uptake system of the gills of freshwater shrimps, which normally steeply absorb salt from freshwater? The first hypothesis that can explain the relative osmotic stability of the hemolymph is that gill ventilation/perfusion would drastically decrease, especially when the exposure is short (up to a few hours). When this happens, consumption of oxygen from the water also decreases. Such decrease in oxygen consumption, however, was not observed in our data. In fact, under all experimental conditions tested here, when a vital dye (methylene blue) was pipetted next to the shrimp, and the branchial chamber and gills were observed under a stereomicroscope, the gills were stained blue (data not shown, see Suppl. material 1). There was great variability in the resulting blue color of the gills of the shrimps. The idea behind this experiment using the blue stain was that, if the shrimp reduced its gill ventilation upon increased water salinity, oxygen consumption from the water would be reduced and hence its gills would remain clear, whitish, when tested. Apparently, however, compatible with the oxygen consumption data, *M. acanthurus* apparently perfuses its gills even under severe salinity increase/stress.

Among estuarine palaemonids, apparent hyporegulation was verified in *Palaemon pandaliformis* (Stimpson, 1871) at 20-30 psu (Freire et al. 2003, Foster et al. 2010) and in *Macrobrachium equidens* (Dana, 1852) at 20-40 psu (Denne 1968). Among freshwater palaemonids, hyporegulation response was observed in the following species of *Macrobrachium*: *M. acanthurus* at 22-26 and 30 psu after 168 hours (Signoret and Brailovsky 2004), at 30 psu after 0.5, 1, 2, 3,6, 16, and 24 hours (Foster et al. 2010), at 25 psu after 24 hours (Maraschi et al. 2015), *M. rosenbergii* at 18-35 psu for 6 hours-15 days (Cheng et al. 2003), *M. brasiliense* at 20 psu, and *M. olfersi* and *M. potiuna* at 20-30 psu for 1-10 days of exposure (Freire et al. 2003). This apparent hyporegulation consists of a response of supression of the hyperegulation, strongly employed in freshwater, in these shrimps, and an still elusive salt secretion, possibly. The response may also be called a hypo-conformation, as in Moreira et al. (1983) for *M.acanthurus* and other species of the genus. This strong tolerance to increases in salinity is in fact expected from the components of a clade that has invaded the freshwater relatively recently (Murphy and Austin 2005, Augusto et al. 2007a, b, 2009, Pileggi and Mantelatto 2010, Collins et al. 2011, McNamara and Faria 2012).

When salinity rises beyond the organisms' homeostatic range of osmoregulation, the osmolality of the hemolymph increases with respect to values in lower salinities but still remains below the osmolality of the water. And this, in turn, beyond a certain limit, leads to an inability to control tissue hydration and volume. The water content in the muscle of *M. acanthurus* was inversely proportional to salinity (strong negative Pearson correlation), and was maintained within a narrow range of variation, with a decrease of ~4-5% in 30 psu with respect to the control (0 psu), after 4-8 hours of exposure. Conversely, at 30 psu, hemolymph osmolality increased by ~50%. This means that, even when the hemolymph experienced a great increase in osmotic concentration, the hydration of the muscle varied very little, indicating that this tissue has high capacity to regulate water concentrations. A similar result was observed in *M. acanthurus* at 30 psu (Foster et al. 2010). The great ability of tissue to maitain its water concentration was also documented for *M. acanthurus* and the other palaemonids *M. potiuna* and *P. pandaliformis*, through an "*in vitro*" experiment in which tissues were exposed to hypo- and hyperosmotic saline solutions that corresponded to a 50% change with respect to the isosmotic control. In this study, the hydration of the shrimp tissues varied in only about 10% (Freire et al. 2013). This high capacity to maintain tissue water levels is in part responsibe for the euryhalinity of *M. acanthurus*, which, throughout its life cycle, switches between freshwater and brackish water (Freire et al. 2008a, 2013)

The maintenance of tissue hydration happens through the regulation of the flux of inorganic ions and concentration of aminoacids or other nitrogenous compounds in the tissues or body fluids (e.g., Pierce 1982, Gilles 1987). Under hyperosmotic challenges, osmolyte concentrations increase in tissues, as already shown for the crustaceans *M. amazonicum*, *M. olfersii*, *Dilocarcinus pagei* (Augusto et al. 2007a, b). As a consequence, aminoacid catabolism should decrease, which is compatible with our results. Thus, the decrease in ammonia excretion observed in our study under hypersaline challenges probably means that there is a mechanism to retain aminoacid or nitrogenous compounds, which allows the maintenance of tissue hydration in high salinities. Compatible with this idea, excretion of ammonia in our results was inversely proportional to salinity (as pointed by the Pearson correlation). The role of aminoacids in the maintenance of tissue hydration has been documented in recent freshwater invaders (Augusto et al. 2007a,b). In summary, when there is an increase in salinity, metabolic energy is routed to controlling extracellular and intracellular homeostasis, by shutting down branchial salt uptake and reducing protein/aminoacid catabolism. The palaemonid shrimp studied here, a recent freshwater invader, does not display "avoidance" or "escape" response when faced with severe salt challenges, even considering its strong and effective apparatus to perform salt uptake from freshwater. An avoidance behaviour would result in reduced oxygen consumption from the water. Rather, although certainly reducing salt uptake, this shrimp maintains

its hemolymph hyposmotic with respect to the ambient water at 20-30 psu for 4-8 hours, during which it uses more oxygen to control water tissue levels . Additional studies are needed to elucidate the limits, degrees, and mechanisms of hypo-regulation or hypo-conformation in palaemonid shrimps.

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LITERATURE CITED

Anger K (2003) Salinity as a key parameter in the larval biology of decapod crustaceans. Invertebrate Reproduction and Development 43(1): 29–45. https://doi.org/10.1080/07924259.2003.9652520

Anger K (2013) Neotropical *Macrobrachium* (Caridea: Palaemonidae): on the biology, origin, and radiation of freshwater-invading shrimp. Journal of Crustacean Biology 33: 151–183. https://doi.org/10.1163/1937240X-00002124

Ashelby CW, Page TJ, De Grave S, Hughes JM, Johnson ML (2012) Regional scale speciation reveals multiple invasions of freshwater in Palaemoninae (Decapoda). Zoologica Scripta 41: 293–306. https://doi.org/10.1111/j.1463-6409.2012.00535.x

Augusto A, Greene LJ, Laure HJ, Mcnamara JC (2007a) Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (Trichodactylidae). Journal of Experimental Zoology A 307: 688–698. https://doi.org/10.1002/jez.a.422

Augusto A, Greene LJ, Laure HJ, McNamara JC (2007b) The ontogeny of isosmotic intracellular regulation in the diadromous, freshwater palaemonid shrimps, *Macrobrachium amazonicum* and *M. olfersi* (Decapoda). Journal of Crustacean Biology 27: 626–634. https://doi.org/10.1651/S-2796.1

Augusto A, Pinheiro AS, Greene LJ, Laure HJ, McNamara JC (2009) Evolutionary transition to freshwater by ancestral marine palaemonids: evidence from osmoregulation in a tide pool shrimp. Aquatic Biology 7: 113–122. https://doi.org/10.3354/ab00183

Bond-Buckup G, Buckup L (1989) Os Palaemonidae de águas continentais do Brasil meridional (Crustacea, Decapoda). Revista Brasileira de Biologia 49: 883–896.

Boudour-Boucheker N, Boulo V, Lorin-Nebel C, Elguero C, Grousset E, Anger K, Charmantier G (2013) Adaptation to freshwater in the palaemonid shrimp *Macrobrachium amazonicum*: comparative ontogeny of osmoregulatory organs. Cell and Tissue Research 353: 87–98. https://doi.org/10.1007/s00441-013-1622-x

Booth, CE, McMahon BR and Pinder AW (1982) Oxygen uptake and the potentiating effects of increased hemolymph lactate on oxygen transport during exercise in the blue crab, *Callinectes sapidus*. Journal of Comparative Physiology 148: 111–121. https://doi.org/10.1007/BF00688894

Bridges CR, Brand AR (1980) The effect of hypoxia on oxygen consumption and blood lactate levels of some marine Crustacea. Comparative Biochemistry and Physiology A 65: 399–409. https://doi.org/10.1007/BF00688894

Charmantier G (1998) Ontogeny of osmoregulation in crustaceans: a review. Invertebrate Reproduction and Development 33: 177–190. https://doi.org/10.1080/07924259.1998.9652630

Cheng W, Liu CH, Cheng CH, Chen JC (2003) Osmolality and ion balance in giant river prawn *Macrobrachium rosenbergii* subjected to changes in salinity: role of sex. Aquaculture Research 34: 555–560. https://doi.org/10.1046/j.1365-2109.2003.00853.x

Claiborne JB, Evans DH, Goldstein L (1982) Fish branchial Na/NH_4 exchange is via basolateral Na, K-activated ATPase. Journal of Experimental Biology 96: 431–434.

Collins PA, Giri F, Williner V (2011) Biogeography of the freshwater decapods in the La Plata basin, South America. Journal of Crustacean Biology 31: 179–191. https://doi.org/10.1651/10-3306.1

Denne LB (1968) Some aspects of osmotic and ionic regulation in the prawns *Macrobrachium australiense* (Holthuis) and *M. equidens* (Dana). Comparative Biochemistry Physiology 26: 17–30. https://doi.org/10.1016/0010-406X(68)90309-5

Foster C, Amado EM, Souza MM, Freire CA (2010) Do osmoregulators have lower capacity of muscle water regulation than osmoconformers? A study on decapod crustaceans. Journal of Experimental Zoology A 313: 80–94. https://doi.org/10.1002/jez.575

Freire CA, Cavassin F, Rodrigues EN, Torres AH, McNamara JC (2003) Adaptive patterns of osmotic and ionic regulation, and the invasion of freshwater by the palaemonid shrimps. Comparative Biochemistry Physiology A 136: 771–778. https://doi.org/10.1016/j.cbpb.2003.08.007

Freire CA, Amado EM, Souza LR, Veiga MPT, Vitule JRS, Souza MM, Prodocimo V (2008a) Muscle water control in crustaceans and fishes as a function of habitat, osmoregulatory capacity, and degree of eurihalinity. Comparative Biochemistry Physiology A 149: 435–446. https://doi.org/10.1016/j.cbpa.2008.02.003

Freire CA, Onken H, McNamara JC (2008b) A structure-function analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry Physiology A 151: 272–304. https://doi.org/10.1016/j.cbpa.2007.05.008

Freire CA, Souza-Bastos LR, Amado EM, Prodocimo V, Souza MM (2013) Regulation of muscle hydration upon hypo-or hyper-osmotic shocks: differences related to invasion of the freshwater habitat by decapod crustaceans. Journal of Experimental Zoology A 319: 297–309. https://doi.org/10.1002/jez.1793

Furriel RPM, Masui DC, Mcnamara JC, Leone FA (2004) Modulation of gill Na+, K+-ATPase activity by ammonium ions: Putative

coupling of nitrogen excretion and ion uptake in the freshwater shrimp *Macrobrachium olfersii*. Journal of Experimental Zoology A 301: 63–74. https://doi.org/10.1002/jez.a.20008

Gilles R (1987) Volume regulation in cells of euryhaline invertebrates. Current Topics in Membrane and Transport 30: 205–247. https://doi.org/10.1016/S0070-2161(08)60372-X

Henry RP, Wheatly MG (1992) Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. American Zoologist 32: 407–416. https://doi.org/10.1093/icb/32.3.407

Maciel JES, Souza F, Valle S, Kucharski LC, da Silva RSM (2008) Lactate metabolism in the muscle of the crab *Chasmagnathus granulatus* during hypoxia and post-hypoxia recovery. Comparative Biochesmistry Phisiology A 151: 61–65. https://doi.org/10.1016/j.cbpa.2008.05.178

Maraschi AC, Freire CA, Prodocimo V (2015) Immunocytochemical localization of V-H$^+$-ATPase, Na$^+$/K$^+$-ATPase, and carbonic anhydrase in gill lamellae of adult freshwater euryhaline shrimp *Macrobrachium acanthurus* (Decapoda, Palaemonidae). Journal of Experimental Zoology A 323: 414–421. https://doi.org/10.1002/jez.1934

Mazzarelli CCM, Santos MR, Amorim RV, Augusto A (2015) Effect of salinity on the metabolism and osmoregulation of selected ontogenetic stages of an amazon population of *Macrobrachium amazonicum* shrimp (Decapoda, Palaemonidae). Brazilian Journal of Biology 75: 372–379. https://doi.org/10.1590/1519-6984.14413

McNamara JC (1987) The time course of osmotic regulation in the freshwater shrimp *Macrobrachium olfersii* (Wiegmann)(Decapoda, Palaemonidae). Journal of Experimental Marine Biology and Ecology 107: 245–251. https://doi.org/10.1016/0022-0981(87)90041-4

McNamara JC, Faria SC (2012) Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: a review. Journal of Comparative Physiology B 182: 997–1014. https://doi.org/10.1007/s00360-012-0665-8

Moreira GS, McNamara JC, Shumway SE, Moreira PS (1983) Osmoregulation and respiratory metabolism in brazilian *Macrobrachium* (Decapoda, Palaemonidae). Comparative Biochemistry Physiology A 74: 57–62. https://doi.org/10.1016/0300-9629(83)90711-9

Murphy NP, Austin CM (2005) Phylogenetic relationships of the globally distributed freshwater prawn genus *Macrobrachium* (Crustacea: Decapoda: Palaemonidae): biogeography, taxonomy and the convergent evolution of abbreviated larval development. Zoologica Scripta 34: 187–197. https://doi.org/10.1111/j.1463-6409.2005.00185.x

Ordiano A, Alvarez F, Alcaraz G (2005) Osmoregulation and oxygen consumption of the hololimnetic prawn, *Macrobrachium tuxtlaense* at varying salinities (Decapoda, Palaemonidae). Crustaceana 78: 1013–1022. https://doi.org/10.1163/156854005775197316

Péqueux A (1995). Osmotic regulation in crustaceans. Journal of Crustacean Biology 15: 1–60. https://doi.org/10.2307/1549010

Pileggi LG, Mantelatto FL (2010) Molecular phylogeny of the freshwater prawn genus *Macrobrachium* (Decapoda, Palaemonidae), with emphasis on the relationships among selected American species. Invertebrate Systematics 24: 194–208. https://doi.org/10.1071/IS09043

Pierce SK (1982) Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solute. Biological Bulletin 163: 405–419. https://doi.org/10.2307/1541452

Rosas C, Ocampo L, Gaxiola G, Sánchez A, Soto LA (1999) Effect of salinity on survival, growth, and oxygen consumption of postlarvae (PL10-PL21) of *Litopenaeus setiferus*. Journal of Crustacean Biology: 244–251. https://doi.org/10.2307/1549230

Salvato B, Cuomo V, Di Muro P, Beltramini M (2001) Effects of environmental parameters on the oxygen consumption of four marine invertebrates: a comparative factorial study. Marine Biology 138: 659–668. https://doi.org/10.1007/s002270000501

Setiarto A, Augusto SC, Takashima F, Watanabe S, Yokota M (2004) Short-term responses of adult kuruma shrimp *Marsupenaeus japonicus* (Bate) to environmental salinity: osmotic regulation, oxygen consumption and ammonia excretion. Aquaculture Research 35: 669–677. https://doi.org/10.1111/j.1365-2109.2004.01064.x

Signoret GP, Brailovsky DS (2004) Adaptive osmotic responses of *Macrobrachium acanthurus* (Wiegmann) and *Macrobrachium carcinus* (Linnaeus)(Decapoda, Palaemonidae) from the southern Gulf of Mexico. Crustaceana 77: 455–465. https://doi.org/10.1163/1568540041643364

Taylor AC, Spicer JI (1989) Interspecific comparison of the respiratory response to declining oxygen tension and the oxygen transporting properties of the blood of some palaemonid prawns (Crustacea: Palaemonidae). Marine and Freshwater Behavior and Physiology 14: 81–91. https://doi.org/10.1080/10236248909378695

Valenti WC, Mello J de TC de, Lobão VL (1989) Fecundidade de *Macrobrachium acanthurus* (Wiegmann, 1836) do Rio de Iguape (Crustacea, Decapoda, Palaemonnidae). Revista Brasileira de Zoologia 6: 9–15. https://doi.org/10.1590/S0101-81751989000100002

Wall SM (1995) Ammonium transport and the role of the Na, K-ATPase. Electrolyte Metabolism 22: 311–317.

Weirauch D, Wilkie MP, Walsh PJ (2009) Ammonia and urea transporters in gills of fish and aquatic crustaceans. Journal of Experimental Biology 212: 1716–1730. https://doi.org/10.1242/jeb.024851

Supplementary material 1

Figure S1. Cephalothorax of *Macrobrachium acanthurus* exposed to salinities 0 (control), 10, 20, and 30 psu for 4 or 8 hours.
Authors: Carolina A. Freire, Leonardo de P. Rios, Eloísa P. Giareta, Giovanna C. Castellano
Data type: JPEG image file
Explanation note: The aquarium water was dyed with methylene blue, coloring the gills and gills chamber according to their ventilatory perfusion. After 5 minutes, shrimps were removed and photographed. An "uncolored" shrimp was included for reference, not exposed to the dye. Despite the variability in the response, it is possible to detect the stain in shrimps exposed to all experimental conditions.
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